Hepatocellular Carcinoma and Polymorphisms in Carcinogen-Metabolizing and DNA Repair Enzymes in a Population with Aflatoxin Exposure and Hepatitis B Virus Endemicity

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Abstract

High rates of hepatocellular carcinoma (HCC) in The Gambia, West Africa, are primarily due to a high prevalence of chronic hepatitis B virus infection and heavy aflatoxin exposure via groundnut consumption. We investigated genetic polymorphisms in carcinogen-metabolizing (*GSTM1*, *GSTT1*, *HYL12) and DNA repair (*XRCC1*) enzymes in a hospital-based case-control study. Incident HCC cases (n = 216) were compared with frequency-matched controls (n = 408) with no clinically apparent liver disease. Although the prevalence of variant genotypes was generally low, in multivariable analysis (adjusting for demographic factors, hepatitis B virus, hepatitis C virus, and TP53 status), the *GSTM1*-null genotype [odds ratio (OR), 2.45; 95% confidence interval (95% CI), 1.21-4.95] and the heterozygote *XRCC1-399* AG genotype (OR, 3.18; 95% CI, 1.35-7.51) were significantly associated with HCC. A weak association of the *HYL1**2 polymorphism with HCC was observed but did not reach statistical significance. *GSTT1* was not associated with HCC. The risk for HCC with null *GSTM1* was most prominent among those with the highest groundnut consumption (OR, 4.67; 95% CI, 1.45-15.1) and was not evident among those with less than the mean groundnut intake (OR, 0.64; 95% CI, 0.20-2.02). Among participants who had all three suspected aflatoxin-related high-risk genotypes [*GSTM1* null, *HLY1**2 (HY/HH), and *XRCC1* (AG/GG)], a significant 15-fold increased risk of HCC was observed albeit with imprecise estimates (OR, 14.7; 95% CI, 1.27-169). Our findings suggest that genetic modulation of carcinogen metabolism and DNA repair can alter susceptibility to HCC and that these effects may be modified by environmental factors.** (Cancer Epidemiol Biomarkers Prev 2005;14(2):373–9)

Introduction

The incidence of hepatocellular carcinoma (HCC) varies greatly worldwide depending largely on variations in the prevalence of known etiologic factors (1). In The Gambia, where annual age-standardized incidence rates exceed 20 cases per 100,000 persons, HCC is the most common cancer in men and second most common in women (2). The high incidence of HCC is associated with high rates of two well-recognized HCC etiologic factors, chronic infection with hepatitis B virus (HBV) and dietary exposure to the mycotoxin, aflatoxin (3). In the Gambia, 15% to 20% of the population are HBV chronic carriers (4, 5), whereas exposure to aflatoxin through the diet is widespread, occurring at all ages and at some of the highest levels observed worldwide (3, 6, 7). Even in regions of sub-Saharan Africa and China with similar widespread exposures to HBV and aflatoxin, there is substantial variation in risk of HCC. Genetic variability may contribute to this observed variation in outcomes at both the individual and population level. Identification of genetic factors related to susceptibility to HCC would help elucidate the complex process of hepatocarcinogenesis and improve the scientific basis for preventive interventions (8-10).

Genes related to aflatoxin metabolism are consequently a primary research interest. Aflatoxin B1 (AFB1), the major aflatoxin that contaminates food, such as groundnuts and maize, is metabolized in the liver by cytochrome *P*450 enzymes to a reactive AFB1-8,9-*exo*-epoxide that can bind to DNA to form the pro-mutagenic AFB1-N7-guanine adduct (10). Glutathione *S*-transferase–mediated conjugation of the reactive 8,9-epoxide to reduced glutathione can protect cellular DNA by preventing adduct formation (11, 12). Genetic polymorphisms in these enzymes may alter the mutagenic and carcinogenic effects of a given level of aflatoxin exposure. *GSTM1* and to a lesser extent *GSTT1* (11, 12) have been implicated in AFB1-8,9-epoxide conjugation and both exhibit a deletion polymorphism resulting in the absence of protein in individuals homozygous for the deletion. In the majority of studies, this null genotype in either *GSTM1* or *GSTT1* was not associated with increased HCC risk or was limited to an effect among subgroups estimated to have the heaviest aflatoxin exposure (13-15). In Taiwan, investigators reported an increased HCC risk associated with aflatoxin biomarkers, limited to individuals with the *GSTM1* or *GSTT1* null genotype (16, 17).

In addition to the glutathione *S*-transferase enzymes, microsomal epoxide hydrolase (*HYL1*) may be involved in the hydrolysis of the AFB1-8,9-epoxide to AFB1-8,9-dihydrodiol, although the experimental evidence has been contradictory (11, 18, 19). The *HYL1**2 allele polymorphism in exon 3 involves substitution of histidine (His) for tyrosine (Tyr) at amino acid position 113 and is associated with a 40%

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decrease of *in vitro* enzyme activity; this effect seems to be related to protein stability (20). The lower activity His variant for *HYL1**2 was associated with an increased risk of HCC in an earlier study in China (21) and one in Italy (22) but not in studies in Sudan (13) and a more recent Chinese study (23).

Once the AFB1-N7-guanine adduct is formed, it may be removed from DNA, either spontaneously or enzymatically, although relatively little is understood concerning the enzymes that repair this adduct. In the case of the X-ray cross-complementing group 1 protein (XRCC1), involved in repair of single-strand DNA breaks, a significant increase in AFB1-DNA adducts in Taiwanese subjects was associated with the *XRCC1*-399 glycine (*XRCC1-399G*) polymorphism (24).

The main aim of the current study was to evaluate factors related to individual susceptibility to HCC in an area with a high prevalence of HCC risk factors. Specifically, individual genetic differences in aflatoxin metabolism and DNA repair enzymes were investigated in HCC cases and healthy control participants well-characterized for HBV, hepatitis C virus (HCV), and 249^{ser} TP53 status, a mutation associated with aflatoxin exposure (3, 10).

Materials and Methods

Study Sites and Population. Details regarding the Gambia Liver Cancer Study, including data on the hepatitis and TP53 status of subjects in this study are reported elsewhere (25, 26). Subject recruitment occurred at three tertiary hospital sites in The Gambia (Royal Victoria Hospital, Banjul; Medical Research Council Hospital, Fajara; Bansang Hospital, Bansang) from September 1997 to January 2001. Briefly, incident cases of HCC (n = 216) were recruited from liver clinics at each site, which evaluated patients with suspected liver disease either referred by local physicians or identified through active surveillance of the wards and clinics. The case definition for HCC included either pathologic confirmation (n = 54, 25.0%) or ultrasonographic liver lesions compatible with HCC and an α-fetoprotein level >100 ng/mL (n = 162, 75.0%). Controls without clinical evidence of liver disease (n = 408) were recruited from the outpatient clinics of the same hospitals, frequency matched by sex and age (within 10 year groupings), and had normal α-fetoprotein levels. Subject evaluation included a structured interview that assessed sociodemographic, lifestyle, and dietary factors; collection of blood and urine samples; and a standardized clinical examination. Institutional review boards from IARC, National Cancer Institute, and Medical Research Council/Gambia Government Joint Ethical Review committees approved the study protocol. Informed consent was obtained from each participant before inclusion in the study.

Laboratory Testing. Blood specimens were processed immediately after collection and stored at −70°C until subsequent testing. Genotyping analysis was done on genomic DNA isolated from lymphocytes using a phenol-chloroform method. An aliquot of this DNA was then shipped to the University of Leeds for genotyping. *GSTM1* and *GSTT1* genotyping was done in a multiplex PCR as previously described with categorization of individuals as having the null genotype (homozygous deletion) or at least one intact allele (27, 28). The *β-globin* gene was amplified as a positive control. The *HYL1**2 (His113Tyr) (20) and the *XRCC1* (Arg399Gln) codon (24) polymorphisms were examined by PCR with restriction enzyme digestion as previously reported.

α-fetoprotein was quantified by standard radiometric assay methods (DiaSorin SA, Sallugia, Italy). HBV surface antigenemia (HBsAg) was determined by reverse passive hemagglutination assay (Murex Diagnostics Limited, Dartford, United Kingdom) with RIA testing of negative samples (Sorin Biomedica Diagnostics, Vercelli, Italy). Anti-HCV status was determined by third generation ELISA (ORTHO, Neckargemund, Germany) with recombinant immunoblot assay confirmation of reactive samples (CHIRON, Emeryville, CA). TP53 249^{ser} mutations were detected in circulating cell-free DNA in plasma using methods previously described (25). Briefly, non-cell-associated DNA was extracted and purified from 200 μL of plasma using standard extraction kits. TP53 249^{ser} mutation was then detected by PCR amplification and restriction analysis and confirmed by direct sequencing of exon-7 PCR products.

Statistical Analysis. Genotype data were analyzed as dichotomous variables (at least one intact allele present versus null) for both *GSTM1* and *GSTT1*. The *HYL1**2 allele polymorphism (Tyr113His) was considered as heterozygote (YH) or homozygote (HH) for the low activity variant His allele. The *XRCC1* polymorphism at exon 10 codon 399 involves a glycine (G) substitution for arginine (A) with a possible reduction in DNA repair with the glycine allele associated with the heterozygote (AG) or homozygote (GG) genotype (24). The primary analysis involved each genotype (*HYL1**2, *XRCC1*) evaluated as a categorical variable with three levels (homozygous low activity, heterozygous, and homozygous high activity). Additional analyses examined combinations of the heterozygous genotypes with the homozygous variant genotype (e.g., YH and HH compared with YY for *HYL1**2). To assess combinations of the genotypes with functional relation to aflatoxin metabolism or activity, a variable was created that included the presence or absence of increasing numbers of low activity genotypes.

Frequency tables of independent variables and genotype data were evaluated for statistical significance by Pearson's χ^2 and Fisher's exact tests. To analyze the risk for HCC associated with each genotype while adjusting for confounders, multivariable unconditional logistic regression was done and odds ratios (OR) along with 95% confidence intervals (95% CI) generated. Adjusted models presented included variables known to vary by case-control status (age, sex, recruitment site, recruitment date, ethnic group, and socioeconomic status variables including education level and living in an earthen floor house) and variables known to be highly associated with HCC (HBV, HCV, and TP53 status; ref. 26). Interaction terms involving each of the primary explanatory variables (HBV, HCV, and TP53) and the polymorphism data were evaluated but none were significant to the $P < 0.100$ level and are not included in the adjusted models (data not shown). Ethnic status was determined by self-report of paternal ethnic group. The three largest ethnic groups in The Gambia are the Mandinka, Fula, and Wollof, which comprised 69.8% of study participants. To maintain evaluable numbers within categories, ethnic groups comprising <10% of the study population were grouped into a single category, the largest of these were the Jola (8.9%) and the Serahule (8.6%). Evaluation of each of the individual genotypes did not reveal any association with TP53 status. Whereas HBV infection is clearly associated with HCC, not all HCC cases will result from HBV-related hepatocarcinogenesis. Similarly, not all HCC cases, even some that are aflatoxin-related, will develop through TP53-mediated pathways. The only significant predictors of TP53 status in this study population were case-control status ($P < 0.001$), season of recruitment ($P = 0.015$), and groundnut intake ($P = 0.05$). Because TP53 status was not directly associated with the genotypes, TP53 was included in the adjusted analysis. The multivariate analysis presented includes models with and without inclusion of TP53 status and represents analysis on the 443 study participants with no missing data. Conditional analysis using similar methods on a postmatched data set comprised by individual matching of HCC cases to one control by age, gender, and site resulted in similar qualitative findings; however, these models were unstable due to decreased sample size (data not shown).

Groundnut intake has limited value as a surrogate for aflatoxin exposure in a case-control study design because of decreased consumption with disease. Despite this, the study participants reported significant groundnut consumption with an average intake of 6.0 servings per week for HCC cases and 6.2 servings a week for controls. To stratify those individuals likely to have the highest dietary AFB1 exposure, groundnut consumption was dichotomized to above the mean versus equal to or below the mean intake of all study participants (6.1 servings per week). Additional analysis for genotype associations with HCC was done, stratifying by potential effect modifiers (groundnut intake and HBsAg status). In a separate case-to-case analysis, the data from HCC cases only were evaluated by similar methods to look for differences in genotype-HCC associations by the primary explanatory variables (TP53, HBV, and HCV status).

Results

The 216 HCC cases were older, more commonly male, and recruited more frequently from the two urban hospitals (Royal Victoria and Medical Research Council Hospitals) compared with the 408 controls (Table 1). Ethnic variation between HCC cases and controls was observed, with more HCC cases reporting Fula (24.2% versus 20.8%) or Wolloff (20.9% versus 15.1%) ethnicity. HCC cases were of lower socioeconomic status, evidenced by a higher proportion of cases living in dwellings with earthen floors or with no formal education (Table 1). Of the controls, 15.9% were HBV chronic carriers and 2.9% were HCV infected. In comparison, 61.1% of HCC cases were HBsAg positive and 18.9% were anti-HCV positive. In adjusted analysis, the ORs for HCC associated with HBsAg or anti-HCV positivity were 21.8 (95% CI, 11.1-43.0) and 15.2 (95% CI, 5.98-38.4), respectively. Plasma 249^{ser} TP53 mutations were detected in circulating cell-free DNA in 39.8% of HCC cases compared with 5% of controls ($P < 0.01$ for all comparisons).

Table 1. Demographic and etiologic characteristics of HCC cases and controls

	Controls*	HCC cases*
	No. (%)	No. (%)
Mean age [SD], y	44.8 [15.2]	48.1 [15.2]
Gender		
Males	292 (71.6)	173 (80.1)
Females	116 (28.4)	43 (19.9)
Recruitment site		
Royal Victoria Hospital	109 (26.7)	85 (39.4)
Medical Research Council	106 (26.0)	68 (31.5)
Bansang Hospital	193 (47.3)	63 (29.2)
Recruitment date		
November-January	100 (24.5)	54 (25.0)
February-April	92 (22.6)	59 (27.3)
May-July	88 (21.6)	50 (23.2)
August-October	128 (31.4)	53 (24.5)
Ethnicity		
Mandinka	132 (32.7)	57 (27.0)
Fula	84 (20.8)	51 (24.2)
Wollof	61 (15.1)	44 (20.9)
Other	127 (31.4)	59 (28.0)
Education		
Ever attended	360 (89.1)	166 (78.7)
None	44 (10.9)	45 (21.3)
Earth floor house		
Yes	200 (49.4)	123 (58.9)
No	205 (50.6)	86 (41.2)
HBV status		
HBsAg negative	338 (84.1)	82 (38.9)
HBsAg positive	64 (15.9)	129 (61.1)
Anti-HCV status		
Negative	371 (97.1)	155 (81.2)
Positive	11 (2.9)	36 (18.9)
Plasma 249^{ser} TP53 mutation		
Absent	336 (96.6)	112 (60.2)
Present	12 (3.5)	74 (39.8)

NOTE: $P < 0.05$ for all comparisons between HCC and control participants.
*Deviations in number of subjects from 216 HCC cases and 408 controls due to missing data.

Because of ethnic variation in our study groups and in HCC rates from The Gambia National Cancer Registry data (2), we evaluated the prevalence of each genotype by ethnic group (Table 2). In evaluation of the controls only, differences in allele frequencies by ethnic classification approached statistical significance only among the *GSTM1* and *XRCC1* genotypes ($P = 0.08$ for both). The prevalence of the *GSTM1* null genotype ranged from 16.0% among the Wollof to 34.2% among the ''other'' category combining the less common ethnic groups. The *XRCC1* homozygous variant allele (GG) was rare in all ethnic groups and absent among Fula and Wollof controls, whereas the heterozygous (AG) allele was most common among the Fula (Table 2).

In univariate analysis, none of the genotypes displayed statistically significant differences in prevalence between HCC cases and controls (Table 3). However, in multivariable analysis with adjustment for age, sex, recruitment site, recruitment date, ethnicity, socioeconomic status, HBV, and HCV status (Table 3), the *GSTM1*-null genotype was associated with a 1.86-fold increased risk for HCC (95% CI, 1.00-3.46). The *XRCC1* AG genotype similarly had a significantly increased HCC risk with an OR of 2.26 (95% CI, 1.06-4.83); there were too few homozygote GG individuals (three controls and three cases) to obtain a meaningful estimate for this genotype. When TP53 status was added to the adjusted model, the observed risk estimates were increased with both genes; ORs were 2.45 for *GSTM1* null (95% CI, 1.21-4.95) and 3.18 for *XRCC1* AG (95% CI, 1.35-7.51). In similar adjusted analysis, the *HYL1**2* HH genotype displayed an almost 3-fold increased risk of HCC but the CI was wide and overlapped unity (OR, 2.83; 95% CI, 0.77-10.4). When risk associated with any variant allele was compared with the homozygous wild type, the findings for each of these genotypes were generally similar to that observed among the heterozygotes alone (Table 3). *GSTT1* status was not associated with HCC.

Stratified by the mean groundnut intake, we observed effect modification of the HCC risk with the *GSTM1* genotype. Whereas no significant association was observed in the lower groundnut intake stratum (OR, 0.64; 95% CI, 0.20-2.02), the *GSTM1*-null genotype had an almost 5-fold increased risk for HCC among individuals consuming at least 6.1 servings of groundnuts per week (OR, 4.67; 95% CI, 1.45-15.1). Effect modification of the other investigated genes by groundnut consumption was not observed.

Because of our previous work suggesting an effect modification of HBV infection on aflatoxin levels and on the effect of aflatoxin-related genes (29-31), we investigated the risk for HCC associated with the genetic polymorphisms separately among HBV carriers and among HBV uninfected participants. However, with all these stratified analyses, the interpretation of the findings and the degrees of significance were greatly limited due to sparse data. Stratified by HBsAg status, we found similar risk estimates of around a 2- to 3-fold increased HCC risk with the *GSTM1*-null genotype and with the combined *XRCC1* AG/GG genotype in both strata (data not shown). The *HYL1**2* HH/HY genotype displayed a 2.5-fold increased OR for HCC among HBsAg-negatives (OR, 2.49; 95% CI, 0.97-6.38) whereas no effect was seen among HBsAg-positives (OR, 0.96; 95% CI, 0.32-2.85).

Table 4 combines the data from the three genotypes [*GSTM1* null, *HYL1**2* (any H allele), and *XRCC1* (any G allele)] postulated *a priori* to be involved in aflatoxin metabolism or

Table 2. Polymorphisms in aflatoxin-metabolizing and DNA repair enzymes by ethnic group

	Mandinka		Fula		Wollof		Other	
	Controls	HCC	Controls	HCC	Controls	HCC	Controls	HCC
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
*GSTM1**								
Present	82 (71.9)	34 (68.0)	59 (76.6)	31 (64.6)	42 (84.0)	29 (74.4)	73 (65.8)	50 (87.7)
Null	32 (28.1)	16 (32.0)	18 (23.4)	17 (35.4)	8 (16.0)	10 (25.6)	38 (34.2)	7 (12.3)
GSTT1								
Present	68 (59.7)	25 (50.0)	41 (53.3)	25 (52.1)	25 (50.0)	19 (48.7)	64 (57.7)	34 (59.7)
Null	46 (40.4)	25 (50.0)	36 (46.8)	23 (47.9)	25 (50.0)	20 (51.3)	47 (42.3)	23 (40.4)
*HYL1*2*								
YY	80 (69.6)	42 (82.4)	56 (72.7)	31 (64.6)	34 (69.4)	28 (71.8)	86 (78.2)	37 (64.9)
YH	28 (24.4)	9 (17.7)	19 (24.7)	13 (27.1)	11 (22.5)	9 (23.1)	19 (17.3)	15 (26.3)
HH	7 (6.1)	0 (0)	2 (2.6)	4 (8.3)	4 (8.2)	2 (5.1)	5 (4.6)	5 (8.8)
XRCC1-399G								
AA	95 (83.3)	46 (90.2)	60 (77.9)	36 (75.0)	44 (88.0)	30 (76.9)	101 (91.0)	48 (84.2)
AG	16 (14.0)	4 (7.8)	17 (22.1)	10 (20.8)	6 (12.0)	9 (23.1)	9 (8.1)	8 (14.0)
GG	3 (2.6)	1 (2.0)	0 (0)	2 (4.2)	0 (0)	0 (0)	1 (0.9)	1 (1.8)

*$P > 0.100$ for all comparisons between HCC cases and controls, except for *GSTM1* null prevalence within the ''other'' ethnic group (P = 0.002).

DNA repair and that our data suggested an increase in HCC risk. Compared to subjects with all functional genotypes present, those with one or two ''high-risk'' genotypes had around a 3-fold increased HCC risk (OR, 2.81; 95% CI, 1.39-5.70 and OR, 3.33; 95% CI, 1.25-8.84, respectively), whereas having all three high-risk genotypes was associated with a 15-fold increased risk (OR, 14.7; 95% CI, 1.27-169). Although we observed an apparent ''dose-response'' effect on HCC risk with an increasing number of variant genotypes present, the risk estimates were imprecisely measured with wide CIs. Subsequently, gene-to-gene effects were examined by estimating HCC risk with combined genotype variables. For *GSTM1* and *XRCC1*, the observed HCC risk with having either one of the high-risk genotypes was 2.15 (95% CI, 1.10-4.20) and it was 9.14 (95% CI, 2.20-38.0) with both high-risk genotypes present compared with neither. When the *GSTM1* null and *HYL1*2* HY/HH genotypes were combined into a single variable, there was no association with HCC for the combined effect, whereas a modest increase in risk was seen with either one present (Table 4). Conversely, with *HYL1*2* HY/HH and *XRCC1* AG/GG genotypes, the combined effect was notably increased compared with the risk with either genotype individually.

As an alternative way to look for effect modifications on the genes of interest by the primary explanatory variables (HBV, HCV, TP53), we did a case-only analysis evaluating the prevalence of each of the polymorphisms stratified by HBV, HCV, or TP53 status. For example, the prevalence of the GSTM1 null genotype was 30.3% among HBsAg-positive HCC cases and was 30.0% among HBsAg-negative HCC cases. Similarly, we did not observe any significant differences for any of the genotypes among HCC cases stratified by the HBV, HCV, or TP53 mutation status (data not shown).

Discussion

In this study, a number of carcinogen-metabolizing (*GSTM1*, *GSTT1*, *HYL1*2*) and DNA repair (*XRCC1*) enzyme polymorphisms were examined among HCC cases and healthy controls from The Gambia, West Africa. Our study found around a 2-fold increased HCC risk with the *GSTM1* null genotype and no effect with *GSTT1* after adjusting for confounders and other clearly defined HCC risk factors (Table 3). Previous studies of the glutathione *S*-transferase family and HCC have yielded mixed results with most studies reporting nonsignificant findings. Positive associations with HCC have generally been weak and limited to the subset of subjects with the highest estimated aflatoxin exposure. In a

Table 3. Genetic polymorphisms in aflatoxin-metabolizing and DNA repair enzymes: prevalence among control participants and HCC cases and estimates of HCC risk

	Controls	HCC cases	Unadjusted HCC risk	Adjusted HCC risk*	Adjusted HCC risk†
	No. (%)	No. (%)	OR (95% CI)	OR (95% CI)	OR (95% CI)
GSTM1					
Present	218 (74.2)	105 (70.5)	1	1	1
Null	76 (25.6)	44 (29.5)	1.20 (0.78-1.86)	1.86 (1.00-3.46)	2.45 (1.21-4.95)
GSTT1					
Present	162 (55.1)	79 (53.0)	1	1	1
Null	132 (44.9)	70 (47.0)	1.09 (0.73-1.61)	1.20 (0.69-2.06)	1.11 (0.61-2.05)
*HYL1*2*					
YY	215 (73.1)	104 (69.8)	1	1	1
YH	66 (22.5)	36 (24.2)	1.13 (0.71-1.80)	1.21 (0.63-2.33)	1.28 (0.62-2.67)
HH	13 (4.4)	9 (6.0)	1.43 (0.59-3.46)	1.55 (0.47-5.09)	2.83 (0.77-10.4)
YH/HH	79 (26.9)	45 (30.2)	1.18 (0.76-1.82)	1.27 (0.70-2.32)	1.51 (0.77-2.94)
XRCC1-399G					
AA	248 (84.4)	120 (80.5)	1	1	1
AG	43 (14.6)	26 (17.5)	1.25 (0.73-2.13)	2.26 (1.06-4.83)	3.18 (1.35-7.51)
GG	3 (1.0)	3 (2.0)	2.07 (0.41-10.4)	1.16 (0.14-9.36)	0.48 (0.04-5.50)
AG/GG	46 (15.7)	29 (19.5)	1.30 (0.78-2.18)	2.11 (1.02-4.36)	2.66 (1.17-6.08)

*Adjusted analysis represents model including age, gender, recruitment site and date, ethnicity, socioeconomic status, HBV, and HCV status.
†Adjusted analysis represents incorporation of TP53 status to above model.

Table 4. Combinations of *GSTM1*, *HYL1*2*, and *XRCC1* polymorphisms and risk of HCC

	Controls	HCC cases	Adjusted HCC risk*
	No. (%)	No. (%)	OR (95% CI)
Combined genotypes†			
No high risk	137 (46.6)	59 (39.6)	1
One high risk	118 (40.1)	64 (43.0)	2.81 (1.39-5.70)
Two high risk	34 (11.6)	24 (16.1)	3.33 (1.25-8.84)
All three high risk	5 (1.7)	2 (1.3)	14.7 (1.27-169)
GSTM1 and *HYL1*2			
Both absent	165 (56.1)	70 (47.0)	1
Either present	103 (35.0)	69 (46.3)	3.34 (1.71-6.49)
Both present	26 (8.8)	10 (6.7)	1.41 (0.37-5.34)
GSTM1 and *XRCC1*-399G			
Both absent	183 (62.2)	86 (57.7)	1
Either present	100 (34.0)	53 (35.6)	2.15 (1.10-4.20)
Both present	11 (3.7)	10 (6.7)	9.14 (2.20-38.0)
*HYL1*2 and *XRCC1*-399G			
Both absent	181 (61.6)	85 (57.1)	1
Either present	101 (34.4)	54 (36.2)	1.53 (0.79-2.98)
Both present	12 (4.1)	10 (6.7)	5.89 (1.36-25.6)

*Adjusted analysis represents multivariable model including age, gender, recruitment site and date, ethnicity, socioeconomic status, HBV, HCV, and TP53 status.

†Genotypes included as high risk (representing low activity) include *GSTM1* null, any H allele for *HYL1*2*, and any G allele for *XRCC1*-399G.

nested case-control study from Taiwan (17), the prevalence of control subjects with detectable aflatoxin-albumin adducts was much lower (40%) than previously observed (>95%) in our population estimates of Gambians (29-31). This near-ubiquitous exposure, coupled to Gambians who have some of the highest levels of exposure in the world (3), causes uncertainty in assessing whether the differences in metabolism from variant genotypes can significantly affect the level of active aflatoxin metabolites. Assessment of aflatoxin exposure in a case-control study is problematic even with biomarkers (10). We attempted to measure aflatoxin exposure in this study using dietary intake of groundnuts as a surrogate measure. Groundnut consumption is a possible surrogate for aflatoxin exposure because this dietary staple is contaminated with high levels of the toxins in The Gambia (32). However, because reported recent groundnut intake was affected by the disease state, there was no association between consumption and HCC risk.[7] Given these limitations (see also Materials and Methods), we only stratified by groundnut consumption for the enzyme systems involved in aflatoxin metabolism and associated with HCC in the overall case-control analysis. Nonetheless, in this population with high aflatoxin exposure, we observed a notable increase in the risk estimate for HCC with the *GSTM1* null genotype among those with the highest groundnut consumption.

Although a slight increase in HCC risk with the *HYL1*2* HH genotype was suggested, the finding did not reach statistical significance in our study (Table 3). McGlynn et al. reported a 3-fold increased HCC risk in subjects with at least one H allele (21) but no association in a more recent study (23). In the first Chinese study, the increased risk with *HYL1*2* genotype was limited to HBsAg-positive subjects. When we did multivariable analysis by HBV status, we observed a 2.5-fold increased risk of borderline statistical significance among HBsAg-negatives but no effect among HBV carriers. An increased HCC risk was previously observed in Sudanese subjects with the HH genotype and high groundnut consumption compared with those with the YY genotype and lower intake (13). In contrast, we did not observe any effect modification by level of groundnut consumption (data not shown).

[7] G.D. Kirk, unpublished data.

Mechanistic evidence for a role of epoxide hydrolase in aflatoxin-related HCC is controversial. It is unclear whether the enzyme would influence the rapid spontaneous rate of hydrolysis of the aflatoxin-epoxide and hence the amount of binding to DNA (12, 18, 19). Nevertheless, the possibility that the enzyme influences HCC risk through an alternative pathway should not be ignored. A previous study of HCV-infected persons in Italy hypothesized that the polymorphism at this locus acted upon endogenous oxidative metabolites due to chronic viral infection rather than exogenous insults (22, 33). The fact that the elevation in risk was present in HBsAg-negative individuals in our study suggests that this pathway may be HCV-specific or that additional mechanisms may also be relevant. It should be noted that whereas HCV was responsible for a large proportion of HBV-negative HCC, the small number of HCV-positive controls limits our ability to assess whether effect modification by HCV status on *HYL1*2* genotype and HCC risk is present or not.

This study also enabled us to evaluate polymorphisms in the DNA repair enzyme, *XRCC1*, with HCC risk. The polymorphism, a glycine for arginine substitution at codon 399 of exon 10, has previously been associated with a functional decrease in DNA repair of single-strand breaks (34) and an increased risk for a variety of cancers (35, 36). This polymorphism was also associated with increased levels of aflatoxin DNA adducts in Taiwanese women naturally exposed to aflatoxin through diet (24). Interestingly, the *XRCC1*-399G allele effect among Taiwanese women was greatest at lower adduct levels (24), possibly indicating that at high exposures, such as those occurring in The Gambia, this repair process may become saturated. We find an increase in HCC risk as hypothesized with the G allele (OR, 3.19; 95% CI, 1.35-7.53; Table 3) but did not find any significant effect modification by the level of groundnut consumption or by HBV status (data not shown). As with the *GSTM1* null genotype, adjustment for plasma TP53 mutation status resulted in increased HCC risk estimates. These findings indicate that both *XRCC1* and *GSTM1* may contribute to HCC pathogenesis either by modulating the effects of other endogenous or exogenous carcinogens or of aflatoxin itself through additional non-TP53-mediated pathways (10).

In another recent report from Taiwan, Yu et al. (37) did not observe an independent increase in HCC risk with *XRCC1*-399G polymorphisms, although a trend was present. However, they found an effect modification of *XRCC1* by glutathione *S*-transferase detoxification genes, with an increased HCC risk limited to *XRCC1*-GG and *GSTT1*-null subjects, whereas no similar effect was observed among *GSTM1*-null subjects.

When high-risk combinations of genotypes were compiled from the polymorphism data, a significantly increased HCC risk was observed with from 1 to 3 of the genotypes linked to aflatoxin metabolism in a dose-response manner (Table 4). Although we had limited statistical power to formally test for interactions, we examined the combined effect of different pairs of genotypes. Polymorphisms of either aflatoxin detoxification enzyme system (*GSTM1*-null or *HYL1*2* HY/YY) combined with a polymorphism affecting DNA repair (*XRCC1*-399G) displayed notably increased risk estimates for the combined effect (Table 4). However, combined-effect ORs with *GSTM1* and *HYL1*2* that, theoretically, may be acting through similar steps in the aflatoxin metabolic pathway were not significant.

The *GSTM1* null, *GSTT1* null, and *HYL1*2* HH genotypes were present in only 27%, 44%, and 5%, respectively, of adult Gambian control subjects, findings consistent with our earlier data in this population (30). This prevalence of the null *GSTM1* genotype is much lower than reported in other African countries, e.g., Sudan (13) and Ghana (21), or in other ethnic groups worldwide (14, 38, 39). The low-activity *HYL1*2* HH genotype prevalence (5%) in Gambians was also

generally lower than Asian and European populations (China, 34%, ref. 21; United Kingdom, 19%, ref. 40; Italy, 15%, ref. 22) but was similar to other African populations (Sudan, 8%, ref. 13; Ghana, 8%, ref. 21).

Data collected over the last decade from the Gambia National Cancer Registry has suggested that the Fula ethnic group may have higher HCC incidence rates compared with the predominant ethnic group, Mandinka (2). Our previous studies showed that aflatoxin-albumin adduct levels were generally lower among Mandinka subjects than either Fula or Wollof subjects (31). In the present study, we identified ethnic variation in the prevalence of genetic polymorphisms, most notably a significantly higher prevalence of the variant Gly allele in the *XRCC1*-399 gene among the Fula (Table 2). Interestingly, we also have seen a higher risk for HCC associated with Fula ethnicity after multivariable adjustment. This raises the possibility that ethnicity could serve as marker of susceptible genotype, although differences in lifestyle also exist between these ethnic groups; for example, the Fula traditionally own more cattle and, therefore, may consume more milk and meat products than other ethnic groups.

There were several limitations to our study. Despite the Gambia Liver Cancer Study being one of the larger studies of HCC reported from Africa, we are still limited by small numbers of subjects in many of the high-risk genotype strata. Inclusion of larger numbers of participants would be helpful in gaining statistical power to better describe subgroup effects, such as for the analysis of gene-to-gene effects or stratified by groundnut status. Although effects of *GSTM1* and *XRCC1* on HCC risk were observed, the CIs were fairly close to 1.0, a frequent observation in studies of low penetrance effects. We did not investigate some other enzyme systems that may be potentially be involved in aflatoxin metabolism, including *CYP1A2*, *CYP3A4*, and *CYP3A5*; these were not included because functional polymorphisms are unidentified or only just being characterized in these genes (11, 41-45).

HCC is a significant health problem in sub-Saharan Africa with poor outcomes and limited treatment options. Prevention of HCC through childhood immunization with HBV vaccine will likely impact HCC incidence rates (46), but it remains unclear if the presence of additional HCC risk factors, including aflatoxin exposure, will limit the impact of vaccination. In addition, the large number of existing chronic HBV carriers remains susceptible to aflatoxin. Several interventions to reduce aflatoxin exposure are possible including chemoprevention (47) and some behavioral practices that may require little direct health expenditures (8). Improved markers of individual susceptibility to aflatoxins will contribute to developing the most appropriate intervention strategies in a given population.

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